

# Chapter 9

## The Nucleocapsid Protein of the SARS Coronavirus: Structure, Function and Therapeutic Potential

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**Abstract** As in other coronaviruses, the nucleocapsid protein is one of the core components of the SARS coronavirus (CoV). It oligomerizes to form a closed capsule, inside which the genomic RNA is securely stored thus providing the SARS-CoV genome with its first line of defense from the harsh conditions of the host environment and aiding in replication and propagation of the virus. In addition to this function, several reports have suggested that the SARS-CoV nucleocapsid protein modulates various host cellular processes, so as to make the internal milieu of the host more conducive for survival of the virus. This article will analyze and discuss the available literature regarding these different properties of the nucleocapsid protein. Towards the end of the article, we will also discuss some recent reports regarding the possible clinically relevant use of the nucleocapsid protein, as a candidate diagnostic tool and vaccine against SARS-CoV infection.

### 9.1 Introduction

By definition, nucleocapsid is a viral protein coat that surrounds the genome (either DNA or RNA). Nucleocapsid protein is the major constituent of a viral nucleocapsid. It is capable of associating with itself and with the genome, thus packaging the genome inside a closed cavity. In some viruses, nucleocapsid protein may also be assisted by other viral cofactors to form the capsid. However, in coronaviruses (including SARS-CoV), the nucleocapsid protein alone is capable of forming the capsid. The primary advantage of the virus for encoding the nucleocapsid protein is that the latter encloses and protects the viral genome from coming into direct contact with the harsh environment in the host. In fact, in some simple viruses

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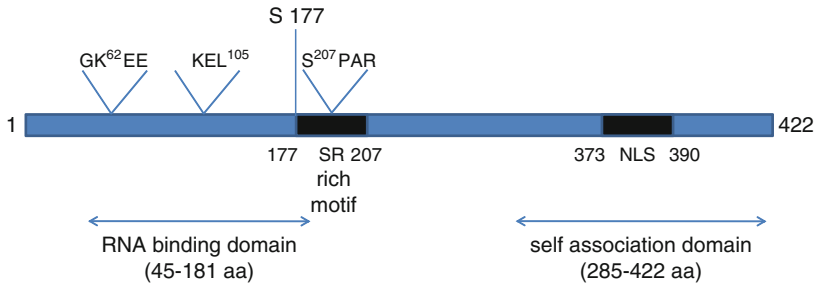
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like hepatitis E virus and polio virus, the nucleocapsid protein is the only coat that protects the genome from the outside world. However, in complex viruses, like hepatitis B virus and coronaviruses (including SARS-CoV), the nucleocapsid is covered by an additional coat composed of other viral proteins (spike protein is a major component of this coat). Besides this property, nucleocapsid proteins of several viruses have been demonstrated to play multiple regulatory roles during viral pathogenesis. They are equipped with specific structural motifs and/or signature sequences, by which they associate with other viral/ host factors and skew the host cellular machinery in such a manner that it becomes more favorable for the survival of the virus. Nucleocapsid protein is also one of the most abundantly expressed viral proteins and it is the major antigen recognized by convalescent antisera. Hence, it is tempting to evaluate its potential as a candidate diagnostic tool or vaccine against the virus.

Therefore, understanding the properties of the nucleocapsid protein is of utmost importance to any virologist in order to understand the biology of the virus and develop effective tools to control the infection. Since the identification and isolation of SARS-CoV in 2003, several laboratories around the world have focussed their research on characterization of various properties of the nucleocapsid protein. An indirect measure of the curiosity among SARS-CoV researchers to study the nucleocapsid protein is revealed from the fact that in PubMed the number of SARS-CoV research publications focussed on nucleocapsid protein is second only to those on spike protein. Evidence accumulated from these articles has helped us gain substantial understanding of the properties of this protein. In this article, we will provide a comprehensive description of all the different properties of the nucleocapsid protein, as established by independent workers from several laboratories. We will conclude this article with the discussion of some of the remaining challenges in this field that need to be addressed in future.

## 9.2 N-Protein: Structure and Composition

The nucleocapsid (N) protein is encoded by the ninth ORF of SARS-CoV. The same ORF also codes for another unique accessory protein called ORF9b, though in a different reading frame, whose function is yet to be defined. The N-protein is a 46-kDa protein composed of 422 amino acids (Rota et al. 2003). Its N-terminal region consists mostly of positively charged amino acids, which are responsible for RNA binding. A lysine-rich region is present between amino acids 373 and 390 at the C-terminus, which is predicted to be the nuclear localization signal. Besides these, an SR-rich motif is present in the middle region encompassing amino acids 177–207. Biophysical studies done by Chang et al. (2006) have suggested that this protein is composed of two independent structural domains and a linker region. The first domain is present at the N-terminus, inside the putative RNA binding domain, and the second domain consists of the C-terminal region that is capable of self-association. Between these two structural domains, there lies a highly disordered



**Fig. 9.1** Structure of the SARS-CoV nucleocapsid protein. A schematic diagram showing different domains identified to date. The numbers 1–422 correspond to the length in amino acids of the *N* gene. GKEE represents the sumoylation motif (lysine residue). KEL is the RXL motif, responsible for binding with cyclin D, and SPAR is the motif that gets phosphorylated by the cyclin–CDK complex (serine residue). S177 is the serine 177 residue that gets phosphorylated by GSK3

region, which serves as a linker. This region has been reported to interact with the membrane (M) protein and human cellular hnRNPA1 protein (Fang et al. 2006; Luo et al. 2005). Besides, this region is also predicted to be a hot spot for phosphorylation. Hence, in summary, the N-protein can be classified into three distinct regions (Fig. 9.1), which may serve completely different functions during different stages of the viral life-cycle. A similar mode of organization has been reported for other coronavirus nucleocapsid proteins.

### 9.3 Stability of the N-Protein

In-vitro thermodynamic studies done by Luo et al. (2004b) using purified recombinant N-protein have shown it to be stable between pH 7 and 10, with maximum conformational stability near pH 9. Further, it was observed to undergo irreversible thermal-induced denaturation. It starts to unfold at 35°C and is completely denatured at 55°C (Wang et al. 2004). However, denaturation of the N-protein induced by chemicals such as urea or guanidium chloride is a reversible process.

### 9.4 Posttranslational Modification

As in other coronavirus N-proteins, SARS-CoV N-protein has been predicted and later experimentally proven to undergo various posttranslational modifications such as acetylation, phosphorylation, and sumoylation.

Acetylation is the first modification of the N-protein to be experimentally proven. By mass spectrometric analysis of convalescent sera from several SARS patients, it has been shown that the N-terminal methionine of N is removed and all

other methionines are oxidized and the resulting N-terminal serine is acetylated. However, the functional relevance of this modification, if any, remains to be elucidated (Krokhin et al. 2003).

Another unique modification of the N-protein is its ability to become sumoylated. Studies done by Li et al. (2005a) have clearly established that heterologously expressed N in mammalian cells is sumoylated. Using a site-directed mutagenesis approach, the sumoylation motif has been mapped to the 62nd lysine residue, which is present in a putative sumo-modification domain (GK<sup>62</sup>EE). Their data further suggests that sumoylation may play a key role in modulating homo-oligomerization, nucleolar translocation and cell-cycle deregulatory property of the N-protein. Further experimental support regarding sumoylation of N-protein came from another independent study carried out by Fan et al. (2006) wherein they have demonstrated an association between the N-protein and Hube9, which is a ubiquitin-conjugating enzyme of the sumoylation system. They have also mapped the interaction domain to the SR-rich motif, which is in agreement with the earlier report. However, they failed to detect the involvement of the GKEE motif in mediating this interaction (Fan et al. 2006).

Initially, the SARS-CoV N-protein was predicted to be heavily phosphorylated. Later on, from results obtained in our laboratory as well as by other researchers, it is now clear that the N-protein is a substrate of multiple cellular kinases. First experimental evidence for the phosphorylation status of the N-protein came from the study done by Zakhartchouk et al. (2005) in which, using [<sup>32</sup>P]orthophosphate labelling, they were able to observe phosphorylation of adenovirus-vector-expressed N-protein in 293T cells. Further studies done in our laboratory clearly confirmed this observation. The majority of the N-protein was found to be phosphorylated at its serine residues (although the involvement of threonine and tyrosine residues could not be detected; they may be occurring *in vivo*). In addition, using a variety of biochemical assays, it was proved that, at least *in vitro*, the N-protein could become phosphorylated by mitogen-activated protein kinase (MAP kinase), cyclin-dependent kinase (CDK), glycogen synthase kinase 3 (GSK3), and casein kinase 2 (CK2). Also, this data provided preliminary indication regarding phosphorylation-dependent nucleo-cytoplasmic shuttling of the N-protein (Surjit et al. 2005). A recent report published by Wu et al. (2008) has further confirmed that N-protein is a substrate of GSK3 enzyme, both *in vitro* and *in vivo*. Using a variety of biochemical and genetic assays, it was clearly demonstrated that serine 177 residue of N-protein was phosphorylated by GSK3. An antibody specific to phospho 177 residue of the N-protein could efficiently detect the phospho N-protein both *in vitro* and in SARS-CoV infected cells. Interestingly, biochemically mediated inhibition of GSK3 activity in SARS-CoV infected cells also leads to around 80% reduction in viral titer and subsequent induction of a virus-induced cytopathic effect. The authors proposed that GSK3 may be a major regulator of SARS-CoV replication, possibly by virtue of its ability to phosphorylate the N-protein. However, phosphorylation of other viral and/or host proteins by GSK3 may also be a determinant of the observed cytopathic effect.

## 9.5 Localization of the N-Protein

In contrast to the N-protein of many other coronaviruses, the SARS-CoV N-protein is predominantly distributed in the cytoplasm, when expressed heterologously or in infected cells (Surjit et al. 2005; You et al. 2005; Rowland et al. 2005). In infected cells, a few cells exhibited nucleolar localization (You et al. 2005). As reported by You et al. (2005), the N-protein contains pat4, pat7 and bipartite-type nuclear localization signals. It has also been predicted to possess a potential CRM-1-dependent nuclear export signal. However, no clear experimental evidence could be obtained regarding the involvement of these signature sequences in regulating the localization of the N-protein. Interestingly, studies done in our laboratory revealed that the majority of N-protein localized to the nucleus in serum-starved cells. This phenomenon could be reproducibly observed both in biochemical fractionation as well as immunofluorescence studies. In addition, treatment of cells with specific inhibitors of different cellular kinases such as CK2 inhibitor and CDK inhibitor resulted in retention of a fraction of the N-protein in the nucleus, whereas GSK3 and MAPK inhibitor had very little effect. Further, N-protein was found to be efficiently phosphorylated by the cyclin-CDK complex, which is known to be active only in the nucleus. The N-protein was also found to associate with 14-3-3 protein in a phospho-specific manner and inhibition of the 14-3-3 $\theta$  protein level by siRNA resulted in nuclear accumulation of the N-protein. Although these experiments are too preliminary to conclusively provide any answer regarding the intracellular localization of N-protein, nevertheless they do provide substantial clues regarding the physical presence of the N-protein in the nucleus, under certain circumstances, which may be a very dynamic phenomenon. Another study done by Timani et al. (2005) using different deletion mutants of the N-protein fused to EGFP showed that the N-terminal of N-protein, which contains the NLS 1 (aa 38–44), localizes to the nucleus, whereas the C-terminal region containing both NLS 2 (aa 257–265) and NLS 3 (aa 369–390) localizes to the cytoplasm and nucleolus. Using a combination of different deletion mutants, they concluded that the N-protein may act as a shuttle protein between cytoplasm–nucleus and nucleolus. Taken together, all these results further suggest that the N-protein per se has the physical ability to localize to the nucleus. Whether this localization is regulated through phosphorylation-mediated activation of a potential NLS or piggy-backing by association with another cellular nuclear protein or through any other mechanism remains to be established.

## 9.6 Genome Encapsidation: Primary Function of a Viral Capsid Protein

Being the capsid protein, the primary function of the N-protein is to package the genomic RNA in a protective covering. In order to achieve this structure, the N-protein must be equipped with two different characteristic properties; such as

(1) being able to recognize the genomic RNA and associate with it, and (2) self-associate into an oligomer to form the capsid. The N-protein of SARS-CoV has been experimentally proven to possess these properties *in vitro*, as discussed below.

### ***9.6.1 Recognition and Binding with the Genomic RNA***

The first experimental evidence regarding the RNA binding property of the N-protein came from the work of Huang et al. (2004), in which, by NMR studies, they proved the ability of the N-terminal domain to associate with several viral 3' untranslated RNA sequences. Additionally, Chen et al. (2007) reported the presence of another RNA binding domain at the C-terminal region (residues 248–365) of the N-protein, which was proposed to be a stronger interaction than that at the N terminus. Based on structural analysis of the RNA–protein interaction, they have further suggested that the genomic RNA is packaged in a helical manner by the N-protein. In another report published by Luo et al. (2006), the RNA binding motif of the N-protein was mapped to amino acid residues 363–382. In summary, the RNA binding ability of the N-protein was attributed to its two distinct structural domains: the N-terminal domain (residues 45–181) and the C-terminal dimerization domain (residues 248–365). These two domains are spatially separated by long stretches of disordered region. A recent study done by Chang et al. (2008) has demonstrated RNA binding ability of these disordered regions. They have proposed that different RNA binding domains of the N-protein may cooperate to enhance the overall RNA binding efficiency of the N-protein and may also serve as interaction hubs for the association of N-protein with other viral and/or host nucleic acid and/or proteins.

Perhaps the most convincing proof to date regarding the ability of the N-protein to package the genomic RNA came from the work of Hsieh et al. (2005). They have established a system to produce SARS-CoV VLPs by cotransfection of spike, membrane, and envelope and nucleocapsid cDNAs into Vero E6 cells. While testing the packaging of an RNA-bearing GFP fused to SARS-CoV packaging signal into this particle, they observed that presence of the N-protein is an absolute requirement. However, the N-protein was not essential for the assembly of the empty particle *per se*. Further, by performing a filter binding assay using recombinant N-protein, they were able to identify two independent RNA binding domains in the N-protein; one at the N terminus (aa 1–235) and the other at the C terminus (aa 236–384). These results are in agreement with previous findings and further suggest that these two regions may be functional *in vivo*. Future experiments using a model infection system will confirm these observations.

### ***9.6.2 Formation of the Capsid***

One of the most crucial properties required by the N-protein for genome encapsidation is its ability to self-associate. Therefore, many laboratories have focused on

characterizing this phenomenon, with an eye on developing possible interference strategies that may help in limiting virus propagation.

Initial studies done in our laboratory using a yeast two-hybrid assay revealed that N-protein is able to self-associate through its C-terminal amino acid 209 residues (Surjit et al. 2004a). A parallel study done by He et al. (2004) using the mammalian two-hybrid system and sucrose gradient fractionation also proved the ability of the N-protein to self-associate to form an oligomer. They further mapped the interaction region to amino acid 184–196 residues, encompassing the SR-rich motif. However, there were some discrepancies regarding the interaction domain mapped in these two studies. Later on, extensive biophysical and biochemical analysis done by Chen's laboratory (Yu et al. 2005, 2006) and Jiang's laboratory (Luo et al. 2006, 2005) have enriched our understanding of the oligomerization process of the N-protein. In summary, the SR-rich motif does possess binding affinity, but this is specific for the central region (aa 211–290) of another molecule of N-protein, instead of the SR-rich motif itself. The C-terminal region (aa 283–422) possesses binding affinity for itself and to associate into a dimer, trimer, tetramer or hexamer, in a concentration-dependent manner. The essential sequence for oligomerization of the N-protein was identified to be residues 343–402. Interestingly, this region also encompasses the RNA binding motif of the N-protein, which prompts us to speculate that there might be mutual interplay between RNA binding and oligomerization activities of the N-protein. Further, the oligomerization was observed to be independent of electrostatic interactions and addition of single strand DNA to the reaction mixture containing tetramers of the N-protein promoted oligomerization. Thus, it has been proposed that once the tetramer is formed by protein–protein interaction between nucleocapsid molecules, binding with genomic RNA prompts further assembly of the complete nucleocapsid structure.

## 9.7 Perturbation of Host Cellular Process by the N-Protein

Besides being the capsid protein of the virus, the N-protein of many coronaviruses is known to double up as a regulatory protein. The N-protein of the SARS-CoV too has been shown to modulate the host cellular machinery *in vitro*, thereby indicating its possible regulatory role during its viral life-cycle. Some of the major cellular processes perturbed by heterologous expression of the N-protein are discussed below.

### 9.7.1 *Deregulation of Host Cell Cycle*

Three different groups have reported the ability of the N-protein to interfere with the host cell cycle *in vitro*. Work done by Li et al. (2005a, 2005b) proved that mutation of the sumoylation motif in the N-protein leads to cell cycle arrest.

Work done in our laboratory has shown the inhibition of S phase progression in cells expressing the N-protein (Surjit et al. 2006). Further, S-phase specific gene products like cyclin E and CDK2 were found to be downregulated in SARS-CoV infected cell lysate, which suggested that the observed phenomenon may be relevant in vivo. In an attempt to further characterize the mechanism of cell cycle blockage induced by the N-protein, several biochemical and mutational analysis were carried out. Results thus obtained demonstrated that the N-protein directly inhibits the activity of the cyclin-CDK complex, resulting in hypophosphorylation of retinoblastoma protein with a concomitant downregulation of E2F1-mediated transactivation. Analysis of RXL and CDK phosphorylation mutant N-protein identified the mechanisms of inhibition of CDK4 and CDK2 activity to be different. Whereas the N-protein could directly bind to cyclin D and inhibit the activity of the CDK4-cyclinD complex, inhibition of CDK2 activity appeared to be achieved in two different ways: indirectly by downregulation of protein levels of CDK2, cyclin E, and cyclin A, and by direct binding of N-protein to the CDK2-cyclin complex.

A third piece of evidence supporting the ability of N-protein to deregulate the host cycle came from the work of Zhou et al. (2008). They observed slower transition from S to G2/M phase and slower growth rate in N-protein-expressing 293T cells. They also observed a similar phenomenon in human peripheral blood lymphocyte and K 562 cells infected with a retrovirus expressing SARS-CoV N-protein.

### ***9.7.2 Inhibition of Host Cell Cytokinesis***

While searching for interaction partners for the C terminus of N-protein (aa 251–422) by following a yeast two-hybrid library screening approach, Zhou et al. (2008) discovered human elongation factor 1 alpha (EF1 $\alpha$ ) as a candidate partner. The specificity of the interaction was confirmed by various in-vitro and in-vivo assays. Further, expression of N-protein induced aggregation of EF1 $\alpha$ . It is known that the majority of cellular EF1 $\alpha$  is bound to F-actin and promotes F-actin bundling, which is a key event during cytokinesis (Kurasawa et al. 1996; Yang et al. 1990). Hence, the authors tested whether N-protein-induced aggregation of EF1 $\alpha$  affected F-actin bundling and cytokinesis. As expected, they observed significantly fewer F-actin bundles in N-protein-expressing cells. In fact, a similar F-actin distribution pattern was also observed by Surjit et al. (2004b) in COS-1 cells. Further, the authors observed multinucleated cells in N-protein-expressing cells at a later time point (72 h post-transfection), indicating inhibition of cytokinesis in those cells. Specificity of the above data has been confirmed by the use of different deletion mutants of the N-protein, in which only the C-terminal domain of the N-protein (responsible for binding with EF1 $\alpha$ ) was able to reproduce the above results. Thus, it has been suggested that EF1 $\alpha$  binding by the N-protein leads to its aggregation, resulting in inhibition of F-actin bundling and subsequent blocking of cytokinesis.



### ***9.7.3 Inhibition of Host Cell Translation Machinery***

EF1 $\alpha$  is known to play a key role during the peptide elongation stage of translation. Therefore, it is an attractive candidate for pathogen proteins to manipulate its activity in order to skew the host translation machinery. For example, HIV-type 1 gag polyprotein has been shown to interact with EF1 $\alpha$  and impair translation in vitro (Cimarelli and Luban 1999). Since Zhou et al. (2008) observed an interaction between EF1 $\alpha$  and SARS-CoV N-protein, they further tested whether it interfered with the host translation machinery. Indeed, presence of the N-protein inhibited total cellular translation, both in vitro and in vivo, in a dose-dependent manner. Moreover, exogenous addition of excess EF1 $\alpha$  could reverse the N-protein-induced translation inhibition, thus suggesting that N-protein exerts its effect by interfering with EF1 $\alpha$  function. However, it remains to be confirmed whether a similar effect is recapitulated in vivo.

### ***9.7.4 Inhibition of Interferon Production***

Production of interferon (IFN) is one of the primary host defense mechanisms. However, SARS-CoV infection does not result in IFN production. Nevertheless, pretreatment of cells with IFN blocks SARS-CoV infection (Spiegel et al. 2005; Zheng et al. 2004). Based on this observation, Palese's laboratory has studied the IFN inhibitory property of different SARS-CoV proteins, which revealed that ORF3, ORF6 as well as the N-protein have the ability to independently inhibit IFN production through different mechanisms. The N-protein was found to inhibit the activity of IRF3 and NF $\kappa$ B in host cells, resulting in inhibition of IFN synthesis. IRF3 activity was also blocked by ORF3, ORF6 proteins, but inhibition of NF $\kappa$ B activity was a property unique to the N-protein. In addition, ORF3, ORF6 proteins were able to block STAT1 activity through different mechanisms (Kopecky-Bromberg et al. 2007). All these data suggest that SARS-CoV may employ multiple factors to check the activity of the host immune system and N-protein may be one of the major partners in this process. It may be possible that these different factors act independently during different stages of the viral life cycle. In that case, regulatory activity of the N-protein will be as indispensable as its structural activity.

### ***9.7.5 Modulation of TGF $\beta$ Signaling Pathway***

During the SARS outbreak, a large number of patients developed severe inflammation of the lungs, which subsequently led to acute respiratory distress syndrome (Ding et al. 2003; Nicholls et al. 2003). Acute respiratory distress syndrome is characterized by pulmonary fibrosis, which results in lung failure and subsequent

death of the patient. The TGF $\beta$  signaling pathway plays a critical role in pulmonary fibrosis (Roberts et al. 2006; Border and Noble 1994). It enhances the expression of extracellular matrix (ECM) proteins, accelerates the secretion of protease inhibitors and reduces the secretion of proteases, thereby leading to deposition of ECM proteins. TGF $\beta$  may also induce pulmonary fibrosis directly by stimulating chemotactic migration and proliferation of fibroblasts as well as by fibroblast–myofibroblast transition. Hence, it is worth speculating that some of the SARS-CoV encoded factors may be modulating the TGF $\beta$  signaling pathway. In fact, proteins of several other viruses, such as hepatitis C virus core, NS3 and NS5 protein, adenovirus E1A, human papilloma virus E7, human T-lymphotropic virus Tax and Epstein–Barr virus LMP1, have been reported to modulate the TGF $\beta$  pathway. In general, these proteins directly bind with smad proteins and alter the innate signaling pathway.

Interestingly, a recent report published by Zhao et al. (2008) revealed that N-protein of SARS-CoV also interacts with smad3 and modulates the activity of the TGF $\beta$  pathway. By performing a smad binding element (SBE)-driven reporter assay, RT-PCR and immunohistological analysis of TGF $\beta$  target genes such as *PAI-1* (plasminogen activator inhibitor 1) and collagen in a variety of cell lines and SARS patients, the authors have clearly proved that N-protein indeed enhanced the activity of the TGF $\beta$  signaling pathway. Further, they observed that the effect of N-protein on TGF $\beta$  signaling was mediated through smad3 only (independent of the involvement of smad4). While trying to unravel the mechanism behind this phenomenon, they observed that N-protein specifically associated with the MH2 domain of smad3 (stronger binding affinity for phospho smad3) interrupted the interaction between smad3 and smad4, and enhanced the interaction between smad3 and transcriptional coactivator p300 in a dose-dependent manner. To further confirm the above data, they performed a chromatin immunoprecipitation assay at the SBE region of *PAI-1* promoter in HPL1 cells and detected the presence of N-protein in the complex of smad3 and p300. Interestingly, however, N-protein inhibited TGF $\beta$ -induced apoptosis of HPL1 cells (it is a well established fact that smad3 activation induces apoptosis of HPL1 cells). Thus, N-protein appears to employ a clever mechanism whereby, on the one hand, it enhances the activity of the TGF $\beta$  signaling pathway, thus leading to enhanced expression of a subset of genes (such as ECM protein coding genes), and on the other hand, it blocks the programmed cell death of the host cell. It would be interesting to unravel the mechanism behind this unique property of the N-protein.

### ***9.7.6 Upregulation of COX2 Production***

Another major proinflammatory factor induced during viral infection is the cyclooxygenase-2 (COX2) protein. Using 293T cells expressing the N-protein, Yan et al. (2006) have shown that expression of the N-protein leads to upregulation of COX2 protein production in a transcriptional manner. They have further demonstrated that the N-protein directly binds to the NF $\kappa$ B response element present in the COX2

promoter through a 68 aa residue binding domain (aa 136–204) and activates its transcription.

Although the N-protein is known to associate with stretches of nucleic acids, to date there is no other documentation or prediction of its sequence-specific DNA binding activity (as a transcription factor). In such a scenario, the above observation, if reproducible *in vivo*, may really be a unique property of the N-protein and may further add to the established regulatory functions of the N-protein.

### ***9.7.7 Upregulation of AP1 Activity***

Exogenously expressed N-protein has been reported to enhance the DNA binding activity of c-fos, ATF-2, CREB-1, and fos B in an ELISA-based assay, thus suggesting an increase in AP1 activity in these cells (He et al. 2003). The mechanistic details and functional significance of this phenomenon remain to be elucidated.

### ***9.7.8 Induction of Apoptosis***

Earlier work done in our laboratory has shown that N-protein, when expressed in Cos-1 monkey kidney cells, induces apoptosis in the absence of growth factors. Attempts to understand the mechanism of programmed cell death revealed that the N-protein downmodulated the activity of prosurvival factors such as extracellular regulated kinase, Akt and bcl 2, and upregulated the activity of proapoptotic factors like caspase-3 and caspase-7 (Surjit et al. 2004b). However, this phenomenon was not observed in another cell line of epithelial lineage (huh7). The above observation was further confirmed by Zhang et al. (2007). They reported that serum starvation-induced apoptosis of N-protein-expressing COS-1 cells involved activation of mitochondrial pathway. Another elegant study done by Diemer et al. (2008) has further extended our understanding regarding the apoptotic property of the N-protein. Through a series of experiments involving both a model infection system of SARS-CoV and transient transfection of N-protein, the authors have confirmed that N-protein induces an intrinsic apoptotic pathway resulting in activation of caspase-9, which further leads to activation of caspase-3 and -6. Their data further revealed that these activated caspases cleave the N-protein at residues 400 and 403 and that nuclear localization of N-protein is an absolute requirement for cleavage. In addition, the authors have reported that the apoptosis-inducing ability of the N-protein is highly cell type specific. Only in cells where N-protein localizes to both nucleus and cytoplasm (Vero E6 and A549 cells), is it able to activate caspase and become cleaved; however, in cell lines where it localizes to the cytoplasm only (Caco2 and N-2a cells), no activation of caspase is observed. It remains to be studied whether this phenomenon is actually recapitulated *in vivo*.

### 9.7.9 Upregulation of Prothrombinase (*hfgl2*) Gene Transcription

A recent report by Han et al. (2008) revealed that, of all the SARS-CoV structural proteins, only N-protein specifically induced the transcription of prothrombinase gene in THP-1 and Vero cells. By performing luciferase reporter assay of *hfgl2* promoter in N-protein-expressing cells and electrophoretic mobility shift assay using N-protein-transfected cell lysate, they demonstrated that N-protein expression induced the binding of transcription factor C/EBP $\alpha$  to its cognate response element present in *hfgl2* promoter, leading to enhanced transcription of *hfgl2* gene. Since lungs of SARS patients have been shown to contain high amount of fibrin, the authors proposed that N-protein-mediated enhanced production of prothrombinase gene may contribute to the development of thrombosis in SARS patients.

### 9.7.10 Association with Host Cell Proteins

Luo et al. (2005) have reported the interaction between hnRNPA1 and N-protein by using a variety of biochemical and genetic assays. The interaction was found to be mediated through the middle region (aa 161–210) of N-protein. If relevant in vivo, this interaction may play a significant role in regulation of the viral RNA synthesis.

Another interesting study done by Luo et al. (2004a) has reported association between the N-protein and human cyclophylin A. By SPR (Surface Plasmon resonance) analysis they have shown it to be a high affinity interaction. Although the significance of this interaction is not known in vivo, they have proposed that this interaction might be crucial for viral infection. Notable is the fact that HIV-1 gag also binds with human cyclophylin A and this interaction is crucial for HIV infection (Gamble et al. 1996).

Recently, Zeng et al. (2008) have reported that N-protein associates with B23, a phosphoprotein in the nucleus. By performing in vivo coimmunoprecipitation in hela cells and GST pull-down assay using purified recombinant N-protein, the authors have demonstrated direct interaction between B23 and N-protein. The interaction domain has been mapped to amino acid residues 175–210 of N-protein, which include the SR-rich motif. B23 plays a key role in centrosome duplication during cell division. Phosphorylation of B23 at threonine-199 residue is known to regulate its function (Okuda et al. 2000, Tokuyama et al. 2001). In order to demonstrate the functional significance of N-protein interaction with B23 protein, the authors tested the phosphorylation status of threonine-199 residue of B23 in the presence of N-protein. Interestingly, N-protein was able to block threonine-199 phosphorylation. Based on this observation, the authors have proposed that N-protein exerts its effect on cell cycle deregulation by modulating the activity of B23 protein.

In summary, although several regulatory roles have been proposed for the SARS-CoV N-protein using a variety of in-vitro experimental systems, no clear evidence exists for their occurrence in vivo. In the absence of a suitable in-vivo experimental system, all these functions remain speculative.

## 9.8 N-Protein: An Efficient Diagnostic Tool

One of the most essential steps to limit the outbreak of any infectious disease is the ability to diagnose the causative agent at the earliest possible time, which can be achieved by detecting some of the markers that are specifically expressed by the pathogen or by identifying some of the host factors that are specifically produced during infection. N-protein, being one of the predominantly expressed proteins at the early stage of SARS-CoV infection, against which a strong antibody response is initiated by the host, has been proposed to be an attractive diagnostic tool.

In serum of SARS-CoV patients, the N-protein has been detected as early as day one of infection by ELISA using monoclonal antibodies against it (Che et al. 2004). Further, a comparative study to detect SARS-CoV-specific IgG, SARS-CoV RNA, and the N-protein during early stages of infection has demonstrated that the detection efficiency of the N-protein is significantly higher than the other two markers (Li et al. 2005b).

Researchers have been mainly focussing on two different strategies by which nucleocapsid can be used as a diagnostic tool: (1) development of efficient monoclonal antibodies against the N-protein, and (2) production of recombinantly expressed, highly purified N-protein for detection of N-protein-specific antibody in the host.

Using a phage display approach, Flego et al. (2005) have identified human antibody fragments that recognize distinct epitopes of the N-protein. These may help develop efficient reagents to detect N-protein in the infected host. Further, several laboratories have been trying to develop efficient monoclonal antibodies against the major immunodominant epitopes of the N-protein, that can be used in ELISA to detect SARS-CoV at an early stage of infection (Shang et al. 2005; Liu et al. 2003; He et al. 2005; Woo et al. 2005). In another interesting study, Liu et al. (2005) have developed an immunofluorescence assay using antirabbit N-protein antibody that can specifically detect N-protein from throat wash samples of SARS-CoV patients at day two of illness.

Several other workers have focussed on economical production of highly purified recombinant N-protein using a variety of heterologous expression systems that can be used in ELISA to detect N-protein-specific antibody in the patient sample. N-protein has been produced in abundant quantity using a codon-optimized gene in *E. coli* (Das and Suresh 2006). Saijo and coworkers have successfully expressed recombinant N-protein using a baculovirus expression system, which was found to be 92% efficient in neutralizing antibody assay (Saijo et al. 2005). In another study, Liu et al. have expressed full length N-protein using a yeast expression system

(Liu et al. 2004). However diagnostic use of recombinant N-protein has been a problematic issue because of several reasons as discussed below.

Bacterially expressed N-protein has been reported to produce false seropositivity owing to interference of bacterially derived antigens (Leung et al. 2006; Yip et al 2007). In addition, several studies have shown cross-reactivity between full-length N-protein of SARS and polyclonal antisera of group 1 animal coronaviruses, which may lead to faulty detection (Sun and Meng 2004). Another study done by Woo et al. also reported cross-reactivity of full-length recombinant N-protein with antisera of HCoV-OC43 and HCoV-229E infected patients, thus giving false positive results. They were able to minimize this false positivity by further verifying the ELISA results with Western blot assay using recombinant N and spike proteins of SARS-CoV (Woo et al. 2004).

Later, studies done by Qiu et al. and Bussmann et al. showed that the recombinantly expressed C-terminal of the N-protein acts more specifically in detecting SARS-CoV-specific antisera in comparison to full-length N-protein (Qiu et al. 2005; Bussmann et al. 2006). It is noteworthy that this region is predicted to encompass major antigenic sites of the N-protein.

In a recent report, Shin et al. (2007) demonstrated significantly higher efficacy of phosphorylated N-protein as a diagnostic antigen. They expressed the N-protein in insect cells, where it was phosphorylated by posttranslational modification. When the antigenicity of this protein was compared to that of a bacterially expressed N-protein (unphosphorylated) or to that of a dephosphorylated N-protein (by treatment with protein phosphatase 1) using SARS-positive or -negative patient serum, phosphorylated N-protein did not show any cross-reactivity with SARS-negative serum, thereby reducing the number of false positives. Also, the phosphorylated protein showed considerably stronger cross-reactivity with an N-protein-specific monoclonal antibody. Based on these observations, the authors have proposed the use of a phosphorylated N-protein as a better diagnostic agent.

Also, several reports have been published dealing with the detection of N-protein-specific IgM by ELISA or indirect immunofluorescent assay (Chang et al. 2004; Hsueh et al. 2004; Woo et al. 2004). However, in these studies, IgM antibodies became detectable later than IgG antibodies, which is in contrast to the phenomena observed in most other pathogens.

A recent report published by Yu et al. (2007) attempted to solve this problem by using a truncated N-protein (aa 122–422) as an antigen in IgM ELISA. They found the IgM response appeared three days before detection of the IgG response, which is in agreement with the results obtained from other known pathogens. Further, their results showed 100% specificity and sensitivity of the truncated protein in detecting N-protein-specific IgM from patients with laboratory confirmed SARS cases in comparison to healthy volunteers. The authors have suggested that the IgM capture ELISA using this truncated N-protein may be more effective in serodiagnosis of SARS-CoV at an earlier time.

In another interesting report, Woo et al. (2005) carried out comparative studies to evaluate the relative diagnostic efficacy of recombinantly expressed N and Spike proteins. They observed sensitivity of recombinant N-IgG ELISA to be significantly

higher than that of recombinant S-IgG ELISA. The reverse was true in the case of IgM ELISA using recombinant N and S proteins. Based on this data, they have suggested the practise of ELISA for detection of IgM against both S and N proteins instead of N alone (Woo et al. 2005).

Taken together, all this data does support the notion that the N-protein may be used as an efficient diagnostic tool for detection of SARS-CoV infection. Nevertheless, production scale-up and further validation of specificity using patient samples will determine the possible clinical use of these reagents.

## 9.9 N-Protein: A Suitable Vaccine Candidate

One of the most clinically relevant uses of the N-protein can be its use as a protective vaccine against SARS-CoV infection. N-protein is one of the major antigens of the SARS-CoV. Also, N-protein analyzed from different patient samples shows least variation in the gene sequence (Tong et al. 2004), therefore indicating it to be a stable protein, which is a primary requirement for an efficient vaccine candidate.

Earlier studies carried in Collins', Rao's, and Li's laboratories have clearly shown that antiserum to the N-protein does not contain neutralizing antibodies against SARS-CoV (Buchholz et al. 2004; Pang et al. 2004; Liang et al. 2005). This may be attributed to the localization of N-protein inside the viral envelope, which will not be accessible to the antibody during infection. It is noteworthy that the most effective SARS-CoV structural protein that can induce neutralizing antibody production is the S-protein (Buchholz et al. 2004). The S-protein antibody could block viral infection with 100% efficiency. On the other hand, although unable to induce humoral immunity, expression of N-protein induced significant cytotoxic T-lymphocyte (CTL) response (Buchholz et al. 2004; Gao et al. 2003; Zhu et al. 2004). Induction of N-protein-specific CTLs will help limit the infection by lysing virus infected cells. This will also limit the spread of virus. Thus, N-protein-based vaccines may further augment the protection efficiency when coadministered with S-protein-based vaccine. Several laboratories have been exploring various strategies to evaluate the potential of N-protein as a vaccine candidate.

In an elegant work done by Kim et al. (2004), calreticulin-fused N-protein expressing vaccinia virus has been shown to generate potent N-protein-specific humoral and T-cell immune responses in mice. As reported by the authors, fusion with calreticulin specifically enhanced the efficiency and significantly reduced the titer of the challenging vector (vaccinia virus). The authors have proposed that N-protein may be the logical choice as a target antigen in the event of S-protein antibody-dependent enhancement (ADE) of infection. However, the ADE phenomenon has not been observed during spike-mediated vaccination (Buchholz et al. 2004). Another study done by Wang et al. (2005) has attempted to use plasmid DNA expressing S, M, and N proteins as an efficient vaccine candidate. Although they report the production of some B-cell and T-cell responses against N-protein,

strong immune response was obtained for the S and M proteins, thus scaling down the choice of N-protein as a suitable candidate vaccine (Wang et al. 2005). A similar plasmid-mediated vaccination approach has also been reported by Zhao et al. (2004), in which they immunized mice with the DNA construct (pCI vector) expressing the N-protein. They too reported the generation of a robust B-cell and T-cell immune response in animals. Another group of workers has also reported successful use of the N-protein as a DNA vaccine. They immunized mice by intramucosal injection of the N-protein-expressing plasmid vector and were able to obtain specific humoral and T-cell responses (Zhu et al. 2004).

The N-protein has also been reported to be of potential interest as a peptide-based vaccine. A systematic study done by Liu et al. (2006) has revealed the immunodominant epitopes of the N-protein which could efficiently stimulate immune response. They have also deduced some conserved immunodominant epitopes in mouse, monkey, and humans, which may help in design of the vaccine.

A recent report published by Gao's laboratory provides further evidence regarding the efficiency of an N-protein-based vaccine (Zhao et al. 2007). By using overlapping synthetic peptides spanning the N-protein, they have identified dominant helper T-cell epitopes in the N-protein of SARS-CoV. Immunization of mice with peptides encompassing these dominant TH cell epitopes resulted in strong cellular immunity *in vivo*. Priming with the helper peptides significantly accelerated the immune response induced by the N-protein. Further, by fusing with a conserved neutralizing epitope from the spike protein of SARS-CoV, two of the TH cell epitope-bearing peptides assisted in the production of higher titer neutralizing antibodies *in vivo*, in comparison to spike epitope alone or its mixture with TH epitope of N. Thus, it is practically possible to generate a better immune response by using a fusion of N and S protein. However, the TH epitopes identified in their report are specific to mouse, and will therefore not be useful for human. Nevertheless, their data provides useful information for the design of peptide-based anti-SARS-CoV vaccines.

Another interesting study conducted by Pei et al. (2005) reports the possible use of the N-protein as a mucosal vaccine candidate. They expressed the N-protein in *Lactobacillus lactis*, which is a food-grade bacteria, and challenged the mice either orally or intramucosally. As preliminary evidence, they were able to observe significant N-protein-specific IgG in the sera of orally challenged animals.

## 9.10 Future Perspective

It is a significant achievement for the research community that, within a short span of time, we have been able to obtain a more-or-less clear understanding regarding the structural and functional properties of the N-protein. However, it is a fact worth mentioning that all the studies done here were performed with *in-vitro* experiments, using recombinantly expressed N-protein, in isolation. So at present, all we can conclude is that the N-protein *per se* has the physical ability to perform the above



described functions, in other words N-protein does bear the necessary signature sequence or motifs or conformation to perform these functions under suitable circumstances. Whether a similar event is recapitulated *in vivo* during viral infection will be dependent on several criteria: (1) the net effect of other viral factors on the activity of N-protein, (2) the net translation and turnover rate of N-protein, (3) a conducive intracellular milieu, and (4) the net modulation of an already skewed cellular pathway by other viral factors. Hence, it will be interesting to reevaluate the properties of N-protein in a SARS-CoV infection model. However, owing to the limited user-friendliness and accessibility of an infection system, we must probably still resort to *in vitro* systems for further analysis of the characteristics of N-protein. One of the better experimental systems has already been established by Chang's laboratory (Hsieh et al. 2005), in which all the structural proteins were coexpressed to form VLP in 293T cells. If this system can be further improved to optimize the rate of synthesis of these different proteins to a level near that *in vivo*, it will at least enable us to study the net effect of the N-protein with respect to other viral proteins. Further establishment of a replicon system may also be helpful. In addition, some of the interesting preliminary observations reported by several laboratories need to be analyzed in detail. To begin with, the reported interaction of the N-protein with the genomic RNA packaging signal needs to be further characterized and mapped. Since the oligomerization domain and the RNA binding regions of the N-protein overlap with each other, the suggested possibility of regulated genome incorporation and capsid assembly should be further characterized with the aid of a replicon system or a particle assembly system. In addition, the reported ability of the N-protein to modulate different cellular pathways should be further characterized in the particle assembly system or at least in the presence of other viral accessory proteins.

The most unique and significant property of the N-protein revealed by preliminary studies is its ability to act as a sequence-specific DNA binding factor. It has been shown to bind the NFκB response element of *COX2* promoter and to enhance *COX2* gene expression. This activity may be further empowering the N-protein to manipulate the entire gene expression programme of the infected cell. Therefore, studies should be initiated to analyze this phenomenon in detail. It seems to deserve so much attention because another study done by Palese's laboratory has proved the ability of the N-protein to inhibit NFκB activity, which results in inhibition of IFN synthesis. Further, Liao et al. (2005) have reported the activation of NFκB by N-protein in Vero E6 cells and He et al. (2005) failed to detect any change in NFκB activity in the same cells. Therefore it needs to be clarified whether N-protein enhances NFκB activity and, if so, whether upregulation of *COX2* transcription by direct DNA binding is a property specific to that promoter or whether it is a global phenomenon. In such a scenario, there may be complicated cross-talk between the ability of N-protein to deregulate the expression of *COX2* and IFN in infected cells.

Lastly, the N-protein is known to be the most abundantly expressed protein of the SARS-CoV. Therefore, any information generated from the analysis of this protein, whether *in vivo* or *ex vivo*, will definitely help to increase our understanding

of the biology of SARS-CoV and may someday help to design better protective tools against it.

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